Sensing Cilia

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Summary & Outlook

Summary

In order to investigate the dynamics of ciliary proteins involved in signal transduction, sensitive (single-molecule) fluorescence microscopy was applied. **Chapter 2** describes the labelling and imaging strategy that was used for most of this work. Proteins of interest were endogenously labeled with a fluorescent protein to be able to observe their dynamics, unperturbed by the unwanted side effects of over-expression. *C. elegans* strains containing fluorescent-protein insertions used in this thesis made by colleagues before me were generated using MoSCI. Strains made by me were made using the CRISPR/Cas9 system. To image the labeled proteins inside living *C. elegans*, a custom-built, laser-illuminated, widefield epifluorescence microscope with sensitive detection was employed. Initial data analysis was usually performed using the ImageJ plugin KymographClear and the stand-alone program KymographDirect. Single-molecule tracking and analysis was performed using custom-written MATLAB routines.

The downside of the setup described in chapter 2, is that the worms have to be mounted on a coverslip. This limits the degrees of freedom to position the sample to three: x, y and z. Although the setup is excellent for sensitive single-molecule imaging of for example intraflagellar transport (IFT) components, the epi-illumination can cause out-of-focus background fluorescence. If one wants to make z-stacks, this background fluorescence decreases the signal-to-noise ratio drastically. To only illuminate the detection objective focal plane, and to be able to position the worm with four degrees of freedom, a light-sheet microscope was constructed (**Chapter 3**). In this instrument, an illumination objective was placed perpendicular to a detection objective. In this configuration, the illumination objective can be used to only illuminate the plane that is in focus of the detection objective, eliminating out-of-focus background fluorescence. Since both objectives are fixed in an objective holder, the stage is used to focus and position the area of interest in the field of view. The stage consists of three accurate closed-loop linear positioning piezomotor stages and a fourth rotational stage, allowing four degrees of freedom. For detection, initially a 100×, N.A. 1.1 Plan achromat objective was used. This objective, however, induced too many optical aberrations, severely affecting image sharpness. The 60×, N.A. 1.0 NIR Apo objective that was implemented in the final design, offered better image sharpness albeit at slightly lower magnification, resolution and collection efficiency. This turned out to be a limiting factor for imaging IFT-

components in cilia at high resolution. We found, however, that the instrument is well suited for volumetric imaging, for example of the *C. elegans* nervous system, allowing recording of three-dimensional movies of a neuronal network in action.

In *C. elegans*, a sensory cilium protrudes from the dendrite of a sensory neuron. As a consequence, the distance between cilium and sites of protein synthesis in the soma is substantial. For ciliary development and function as chemosensor, IFT components continuously cycle from the ciliary base to the tip and back again. **Chapter 4** investigates how dendritic input of ciliary components affects IFT and ciliary function. The real-time response of IFT components to femtosecond laser ablation of the dendrite was visualized using fluorescence microscopy. We found that the response occurs in three stages. First, IFT dynein is activated within seconds, redistributing IFT components towards the ciliary base; second, the ciliary axoneme shortens and motors slow down; and third, motors leave the cilium. Additional experiments show that activation of retrograde transport is not triggered by ATP depletion. Taken together, these results indicate that laser ablation triggers a specific mechanism allowing the IFT system to rapidly adapt to environmental cues.

At the forefront of sensing, the tip of the cilia is an intriguing site. At the tip, anterograde trains arrive, and retrograde trains leave to return to the base. To investigate what happens during this turnaround, we performed single-molecule imaging of four key IFT components: OSM-3, IFT-A component CHE-11, IFT-B component OSM-6 and IFT-dynein (XBX-1) (**chapter 5**). Our data show that OSM-3, IFT-A and IFT-dynein turn around almost instantly after arrival at the tip. IFT-B component OSM-6 however, is retained at the tip for a short while, possibly undergoing modification before joining a retrograde train. This indicates that the anterograde IFT-trains disassemble into at least the IFT-A, IFT-B, IFT-dynein and OSM-3 complexes before returning to the base. Taken together, the SM-approach as employed in this chapter is a valuable tool to dissect directional switches in bidirectional intracellular transport driven by opposite-polarity, microtubule-based motors.

C. elegans chemosensory cilia are at the forefront of the signal-transduction cascade that can lead to behavioral change. To assess the real-time response of involved ciliary components, we exposed *C. elegans* expressing fluorescently labeled tubulin, the molecular motor IFT-dynein, and the transmembrane calcium-channel OCR-2 to aversive

chemicals (**chapter 6**). The experiments demonstrate a remarkable, robust and reversible redistribution of ciliary components out and into the ciliary distal segment. Genetically severing the link between OCR-2 and IFT shows that OCR-2 relies on both active transport by IFT and diffusion for its steady-state ciliary distribution. Single-molecule fluorescence imaging of OCR-2 was performed to elucidate the mechanisms underlying the steady-state distribution and reversible redistribution. From advanced analysis of the single-molecule trajectories, the picture arises that OCR-2 distribution and dynamics are governed by location-specific motility of OCR-2: OCR-2 is mostly transported by IFT in dendrite and transition zone, while diffusion in the ciliary membrane is much more prominent further along the cilium, with mostly normal diffusion in the proximal segment and sub-diffusion in the distal segment. At the ciliary tip, OCR-2 is mostly static, possibly held in place by interactions with other proteins. Taken together, the data demonstrates an intricate interplay between OCR-2's transportation modes that enable it to function as ciliary signal transducer. On a broader scale, these insights into the dynamics of ciliary signal transduction components contribute to a wider understanding of IFT dynamics and to cilia as chemosensory organelles.

Outlook

By employing several advanced fluorescence-microscopy techniques, I sought in this thesis, to detect, observe, perturb and elucidate the mechanisms of intraflagellar transport and ciliary signal transduction in live *C. elegans*. Combined, the work presented here contributes to this fascinating field, and apart from providing new hypotheses it also poses new, intriguing questions. Before elaborating on biological questions raised by the work in this thesis, I will first comment on possible methodological advancements.

Faster imaging

Although my single-molecule fluorescence experiments have provided insights that would have remained elusive in ensemble imaging, intriguing dynamics and structures undoubtedly await below the time window of 100 ms and a localization accuracy of 40 nm that was used in this thesis. The obvious consequence of reducing the exposure time, while keeping the excitation intensity constant, is a reduction in signal-to-noise ratio (SNR). Instead of employing better probes and more sensitive instrumentation, increasing excitation power would be the easiest way to increase SNR. Recent experiments with GFP labeled nematodes in our laboratory have shown that increasing excitation laser power does allow for an increase of the time resolution to up to 50 ms, though at a cost. Higher laser power results in faster bleaching of fluorescent probes, thereby decreasing the duration over which foci are distinguishable. This also means that focusing on those few not-yet-bleached foci, becomes increasingly harder. Nevertheless, with considerable time and effort, it is feasible to obtain a dataset of single-molecule trajectories with an exposure time of 50 ms, revealing, for example, what lies beyond the 150 ms exposure time of the tip turns of chapter 5.

Better probes

Although an exposure time of 50 ms might not be the lower limit of eGFP, other probes might outshine its capabilities. The ideal probe should be non-bleaching, have a high fluorescence quantum yield, and be possible to target to specific proteins of interest. New fluorescent proteins and synthetic fluorophores are constantly being developed (Cranfill et al., 2016; Grimm et al., 2017; Heppert et al., 2016; Zheng and Lavis, 2017), however, inside living organisms like *C. elegans* they often do not live up to their

expectations. Functionalized quantum dots (QDs) might be able to fulfill the criteria mentioned above (Vu et al., 2015). The work of E. Katrukha and collegues (2017) is an elegant example of the use of QDs for high time resolution (50 ms) imaging of motor proteins. The bright fluorescence allowed them to obtain a localization precision of 3-4 nm (Katrukha et al., 2017). Although a large Stokes shift and far-red emission make QDs ideal for *in vivo* imaging, getting funcitionalized QDs inside *C. elegans* cilia might provide a major challange. Click-chemistry, employing the strong binding of for example biotin and streptevadin, could be used to target biotinilated QDs to a streptevadin-labeled protein of interest. A first step would be to genetically label ones protein of interest with streptavidin, providing the specificity for biotinilated QDs. Although endocytosis is known to take place around the ciliary base, QDs taken up via this route can end up in endosomes, rendering them useless (Clement et al., 2013; Pedersen et al., 2016). Also coating functionlized QDs with cell-penatrating coatings like diseleno lipoic acid and cell-penetrating poly(disulfide)s, seem to resolve this problem and reliable deliver QDs into the cytosol (Bartolami et al., 2019; Derivery et al., 2017). When studying transmembrane proteins like OCR-2, QDs could also be attached to a streptevadin-tagged extracellular part of the protein, circumventing the need to import the QDs inside the cell. This stratagy has been successfully used before, though not in multicellular organisms (Ye et al., 2018). Despite the major hurdle of delivering QDs into *C. elegans* cilia, once taken, it would be possible to obtain long (> 20 s) trajectories with high (<50 ms/frame) framerate, high localization precision (<40 nm) and high Signal-to-Background Batio (SBR).

Harder objects

Besides micrometers of tissue that induces optical aberrations, and auto fluorescence of surrounding tissue that decreases SBR, the expression of fluorescently labeled proteins in surrounding tissue limits SBR in multicellular organisms. This is a major reason why the work in this thesis focuses on *C. elegans* phasmid cilia. Contrasting to the four cells that harbor those chemosensory cilia, the majority of the surrounding cells are not ciliated or neurons, and express different variants of intracellular transport proteins. Therefore, they do not express fluorescently labeled proteins. The other chemosensory cilia in *C. elegans* reside in its head region, surrounding the pharynx. On top of auto fluorescence coming from the cuticle and pharynx, most IFT proteins are expressed in

all those 30 cilia. They are situated close together, therefore decrease SBR and make distinguishing between cilia hard. The split-GFP approach would provide a solution for this problem (Cabantous et al., 2005). By splitting the 11 β-strands of GFP in two fragments, only cells expressing both fragments will have functional GFP, allowing for cell-specific fluorescence labeling (Cabantous et al., 2005; Feng et al., 2017). As worm strains with somatic expression of GFP_{1-10} are publicly available, the gene of interest only has to be labeled with the short DNA fragment of GFP_{11} (Kenyon and Ingaramo, 2020). As insertion efficiency scales with a decrease of insert size, the split-GFP method should allow for more efficient generation of endogenous strains, and increase SBR when emission from surrounding cells would hamper SBR.

Beyond epifluorescence

Custom-built, laser-illuminated epifluorescence setups, built around a Nikon body, with a sensitive camera and high (1.49) NA objective allowed me to capture single-molecule behavior in a living multicellular organism. As mentioned above, high out-of-focus fluorescence reduces SBR drastically, especially in the head region. This was a major reason to design and build a light-sheet microscope (LSM). Although it performed well during volumetric imaging experiments of soma's expressing the calcium sensor GCaMP, the straightforward setup turned out to be no match for our epifluorescence setups for imaging IFT. Mostly the sample embedding in agarose and lower NA, water-dipping objectives are to blame for the lack in performance.

So how would the next generation of microscopes in our lab look like? Next to straightforward updates of the cameras and objectives of the existing epifluorescence setups, more could be achieved by building a more elaborate LSM. Improvements on the current LSM could be made in two areas: sample preparation and type of light-sheet. Upright positioning of the two, perpendicular orientated, objectives would allow for convenient and manipulable positioning of *C. elegans* on a horizontal surface. To minimize aberrations due the sample preparation, and prevent movement of the nematodes, they should be anaesthetized and either placed under a very thin agarose pad, or be glued to the glass surface. Contrasting to our Gaussian light sheet, Bessel beams are thin and uniform in their propagation direction. Though the formation itself is straightforward, the result is a long and narrow circular beam, not a sheet of light. A virtual sheet has to be created by a scanning mirror. Though this increases setup

complexity, the resulting thin and consistent light sheet is a major improvement on the Gaussian light sheet, which is almost twice as wide at the sides of the FOV compared to the center.

Besides possible technical advancements, my thesis prompts various biological questions raised by my research and that of others.

Only friends can enter

The gate of the cilium does not grant access to every protein (Garcia-Gonzalo and Reiter, 2017). The dense protein composition of the transition zone (TZ) and its membrane only allow small proteins (≲100 kDa), and larger proteins that are ferried across by the BBSome, likely granted access by a ciliary localization signal and escorted out by the BBSome after ligand mediated activation (Harris et al., 2020, 2016). In chapter 6, I show that no IFT-mediated TZ crossings are observed for the transmembrane protein OCR-2 in a BBSome mutant background. Even though, OCR-2 signal is present in these cilia. This is peculiar, since I have never seen OCR-2 crossing the TZ by diffusion. Though without OCR-2's connection to the motor proteins via the BBSome, and the IFT-A and IFT-B particles, the only way in for OCR-2 is by diffusion. It would be most interesting to find out which protein(s) form the link between OCR-2 and the IFT-trains. The single-molecule imaging showed that this is a transient connection, as stretches of actively transported OCR-2 are interspersed with ones of diffusion. Simple mutation studies combined with single-molecule imaging of the TZ would be a good start. A likely candidate for OCR-2's connection with the BBSome is TUB-1, the *C. elegans* TULP3 homolog, which is essential for ciliary import of some other ciliary transmembrane proteins (Mukhopadhyay et al., 2010). Along these lines, it would also be interesting to investigate what intracellular part(s) of transmembrane proteins like OCR-2 are connected to the BBSome/IFT trains.

There and back again

As the tip of the axoneme forms the dead end of the cilium, what comes in, must, at some point, also return and come out. Apart from *i.a.* GPCRs going on an adventure and escaping the cilium altogether via ectosomes shedding from the ciliary tip (Nager et al., 2017), the majority of proteins leave the cilium via retrograde transport. Chapters

4, 5 and 6 touch upon the transformation of anterograde trains into retrograde trains. Although recent structural work has shown that IFT dynein spans out over seven to eight IFT-B repeats, which reinforces its auto-inhibition during anterograde transport, the trigger for its activation remains elusive (Toropova et al., 2019). Studies seeking to induce IFT-train turnaround in a more specific way than shutting off dendritic ciliary input might provide answers. Interesting experimental tools could include light-inducible dimerization or ubiquitination of IFT-components, specific light-inducible small molecule inhibitors and microfluidics (Chronis et al., 2007; Döbber et al., 2017; Harterink et al., 2016; Hermann et al., 2015).

In chapter 5, I showed that IFT-B particles pause during turnaround at the ciliary tip. It has also been shown that IFT-B is enriched upon GPCR activation together with the BBSome in an Arl6-dependent manner, which allows for ciliary exit of GPCRs by retrograde IFT-trains (Ye et al., 2018). Together with the finding of IFT-dynein's auto inhibition by IFT-B during anterograde transport, it strongly suggests that IFT-B is remodeled before it returns to the base. So far, affinity purification and mass-spectrometry data suggests there is only one physical link between the IFT-B particle complex and the BBSome: the C terminus of IFT38 (*C. elegans* dyf-3) (Beyer et al., 2018; Chou et al., 2019; Nozaki et al., 2019). Exploring the details of this link by mutations in the C terminus of IFT38 might provide answers to the nature of IFT-B remodeling and its connection with the BBSome. Furthermore, as more IFT-train ultrastructures are resolved, we may find clues in the different conformation of anterograde and retrograde IFT trains. As IFT-B is a particle complex, functional studies and single-molecule imaging of its numerous subunits will also likely yield answers to the molecular mechanisms of IFT-B remodeling.

Integrated view on the response to chemical ques

A major question coming from the repellent experiments of chapter 6 is what causes the delay between the addition of the stimulus and the redistribution of ciliary components, and what the trigger for this redistribution is. Does the signal go to the soma and back to the cilium before it causes IFT components to redistribute? Or does the delay happen in the cilium itself? It would also be interesting to study the possible habituation effect. Will a signal be still transduced during sequential exposure to repellents? Can a signal be transduced when IFT components are not at the tip of the cilium anymore?

Performing repellent experiments while simultaneously observing the response in compartments beyond the cilium would create a more integrated view on the functional relationship of IFT dynamics and sensory signal transduction. This could be performed in microfluidic devices that allow for time-controlled addition of chemicals whilst allowing sensitive fluorescence imaging (Chronis et al., 2007). Although their position in Z is different, imaging GCaMP in the soma's simultaneously with an IFT component like IFT-B in the phasmid cilia would be a good place to start. Besides using GCaMP to look at calcium, one could label and image other second messengers, like cAMP and G proteins, that transduce signals from the cilia to the soma. These methods could for example provide more insight into how and why the reversible redistribution of IFT components upon stimulation occurs on the many seconds timescale we observed.

Kap-1 mutant axoneme structure and MIPs

The earlier finding from our lab that the lack of Kinesin-II deforms the axoneme, raises intriguing questions on the relationship between IFT trains and the structure of the axoneme (Oswald et al., 2018). Using light microscopy to locate the phasmid cilia in the worm prior to electron microscopy (EM) using a correlative light and electron microscopy approach, could provide valuable structural information that might give a clue to the nature and cause of this deformation (Burel et al., 2018; de Boer et al., 2015).

EM on the doublet microtubules of *Tetrahymena* cilia, and *Chlamydomonas* and sea urchin sperm flagella allowed for the discovery of microtubule inner proteins (MIPs) (Ichikawa et al., 2019; Ma et al., 2019; Nicastro et al., 2011, 2006). Although few MIP genes have been identified and no *C. elegans* MIPs are known to date, they seem important for doublet microtubule structure and stability. Therefore, MIPs might be implicated in IFT regulation, axoneme dynamics, and be involved in the axonemal deformation of the kap-1 mutant.

All these questions and potential ways to address them demonstrate the advancement of science, but also indicates we barely scratched the surface of this intriguing field, to which my thesis is a humble contribution.

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